Binding to and Inhibition of Insulin-Regulated Aminopeptidase by Macrocyclic Disulfides Enhances Spine Density

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ABSTRACT

Angiotensin IV (Ang IV) and related peptide analogs, as well as nonpeptide inhibitors of insulin-regulated aminopeptidase (IRAP), have previously been shown to enhance memory and cognition in animal models. Furthermore, the endogenous IRAP substrates oxytocin and vasopressin are known to facilitate learning and memory. In this study, the two recently synthesized 13-membered macrocyclic competitive IRAP inhibitors HA08 and HA09, which were designed to mimic the N terminus of oxytocin and vasopressin, were assessed and compared based on their ability to bind to the IRAP active site, and alter dendritic spine density in rat hippocampal primary cultures. The binding modes of the IRAP inhibitors HA08, HA09, and of Ang IV in either the extended or γ-turn conformation at the C terminus to human IRAP were predicted by docking and molecular dynamics simulations. The binding free energies calculated with the linear interaction energy method, which are in excellent agreement with experimental data and simulations, have been used to explain the differences in activities of the IRAP inhibitors, both of which are structurally very similar, but differ only with regard to one stereogenic center. In addition, we show that HA08, which is 100-fold more potent than the epimer HA09, can enhance dendritic spine number and alter morphology, a process associated with memory facilitation. Therefore, HA08, one of the most potent IRAP inhibitors known today, may serve as a suitable starting point for medicinal chemistry programs aided by MD simulations aimed at discovering more drug-like cognitive enhancers acting via augmenting synaptic plasticity.

Introduction

In 2001, the hexapeptide angiotensin IV (Ang IV; Val-Tyr-Ile-His-Pro-Phe), which improves memory and learning in both rats and mice (Braszko et al., 1988; Wright et al., 1993, 1996, 1999; Lee et al., 2004; Gard et al., 2007, 2012; Braszko et al., 2008; De Bundel et al., 2009), was demonstrated to inhibit the membrane-bound zinc-dependent insulin-regulated aminopeptidase (EC 3.4.11.3 IRAP, oxytocinase), a member of the M1 family, which is expressed in a diversity of tissues/cells (Albiston et al., 2001; Liao et al., 2006; Saveanu and van Endert, 2012; Nikolaou et al., 2014). There has been dispute in the field as to the target that mediates the memory-enhancing effects of Ang IV, because this hexapeptide has the ability to bind to other proteins (Vanderheiden, 2009; Kawas et al., 2012; Benoist et al., 2014; Wright and Harding, 2015). Nevertheless, the hypothesis that Ang IV and its analogs improve cognitive function through IRAP inhibition by altering levels of neuropeptides in the brain and/or exerting their actions by facilitating glucose uptake in neurons have attracted the most attention (Vauquelin et al., 2002; Albiston et al., 2003; Chai et al., 2004). There is a need for improved cognitive enhancers for the treatment of Alzheimer’s disease and related disorders and it is therefore not surprising that considerable efforts have been devoted to the discovery of compounds that can mimic the positive effects of Ang IV (Wolfe, 2002; Chai et al., 2008; Gard, 2008; Wright et al., 2008, 2015; Hallberg, 2009; Kawas et al., 2012; McCoy et al., 2013). Both potent and specific drug-like

ABBREVIATIONS: Ang IV, angiotensin IV; Ang IV-gt, Ang IV complex with the inverse γ-turn conformation; ANOVA, one-way analysis of variance; AP-N, aminopeptidase N; DIV, days in vitro; ΔGbind, binding free energies; IRAP, insulin-regulated aminopeptidase; LIE, linear interaction energy; LTA4H, leukotriene A4 hydrolase; LTP, long-term potentiation; MD, molecular dynamics; PDB, Protein Data Bank; vGLUT1, vesicular glutamate transporter 1.

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IRAP inhibitors (Borhade et al., 2014; Mountford et al., 2014), some with proven effects in vivo (Albiston et al., 2008, 2011), and inhibitors with more peptidic character (Kobori et al., 1997, 1998; Wolfe, 2002; Axen et al., 2006; Axen et al., 2007; Andersson et al., 2008; Lukaszuk et al., 2008, 2009; Hallberg, 2009) have been disclosed by us and others. We use an approach to discover efficient Ang IV peptidomimetics that relies on stepwise alterations of Ang IV by applying various cyclization procedures to restrict conformational flexibility and to allow determination of the bioactive conformation of Ang IV when it binds to IRAP (Axen et al., 2006, 2007).

Endogenous IRAP substrates, such as the macrocyclic peptides oxytocin and vasopressin (Fig. 1), that encompass a disulfide element adjacent to the cleavage site of the ring system are known to exert favorable influences on cognition (Chai et al., 2004; Stragier et al., 2008). Our medicinal chemistry approach has taken advantage of this knowledge to develop potent macrocyclic IRAP inhibitors (Andersson et al., 2010, 2011).

Previous studies have observed a correlation between the prococeptive effects of Ang IV-derived peptides in rodents and their capacities to increase spine number and alter morphology (Benoist et al., 2011; McCoy et al., 2013). These data suggest that the positive impact of these peptides and peptidomimetics on cognition and dendritic spines might be attributed to a possible capacity to suppress the catalytic activity of the aminopeptidase IRAP.

We selected two macrocyclic competitive IRAP inhibitors that differ only with regard to one stereogenic center (Fig. 2), one exhibiting powerful inhibitory activity (HA08) and one that is 100-fold less potent (HA09). In this study, binding modes of the peptide inhibitor Ang IV, the macrocyclic inhibitors HA08 and HA09, and the cyclic substrate oxytocin, which is specifically hydrolyzed by IRAP, are presented by means of a computational analysis starting from the recently reported three-dimensional structure of human IRAP (Hermans et al., 2015). We herein report that the IRAP inhibitor HA08, in contrast to the structurally similar, but less potent, IRAP inhibitor HA09, binds more favorably to the active site of IRAP and increases dendritic spine number, which provides an argument for the proposal that specific inhibition of IRAP activity accounts for the observed outcome. From this study, which includes molecular dynamics and binding free energy calculations, we envisage the structural determinants of the differences in activities experimentally reported between the different inhibitors.

**Materials and Methods**

**Syntheses**

The synthesis of HA08 and HA09 was reported previously (Andersson et al., 2010). The two macrocycles were prepared by manual solid phase peptide synthesis using the fluorenlymethoxycarbonyl chloride protection strategy followed by oxidative cyclization in solution.

**Structural Modeling and Molecular Docking**

The crystal structure of human IRAP was retrieved from the protein data bank (PDB code: 4PJ6) (Hermans et al., 2015). The crystal cell is constituted by a dimer and we retained chain A for our calculations, including protein atoms and the Zn$^{2+}$ ion. Missing side chains and hydrogens were added with the pdb2pqr tool in CHIMERA (Petterson et al., 2004). The binding mode of Ang IV in its extended conformation was modeled by superimposing onto the crystallographic structure of Ang IV in complex with the homologous human aminopeptidase N (AP-N) (Laustsen et al., 2001; Wong et al., 2012; Hermans et al., 2015) with PDB code 4FYS (Wong et al., 2012). The initial model of the IRAP-Ang IV complex with the inverse γ-turn conformation (Ang IV-gt) of the peptide (Axen et al., 2006; Andersson et al., 2010; Andersson and Hallberg, 2012) was created by adjustment of the corresponding dihedrals at the proline residue using the PyMol Molecular Graphics System, version 1.5.0.4 (Schrödinger, LLC; NY). Ligands HA08 and HA09 (Andersson et al., 2010) were modeled using Maestro, version 9.2 (Schrödinger, LLC) followed by energy minimization and a conformational search with MacroModel, version 9.9 (Schrödinger, LLC), using the OPLS-2005 (Jorgensen et al., 1996) force field and a root mean square deviation cut-off of 1.0 Å for clustering. All the resulting ~50 conformers were docked independently with GLIDE (Halgren et al., 2004), using XP precision on a 30-Å grid centered on the Co position of His4 in Ang IV (as docked to IRAP above). The top-10 ranked poses for each inhibitor were refined with 1 ns molecular dynamics (MD) simulations (see parameters below). Within these, we retained the binding pose that was more compatible with the binding interactions modeled for Ang IV, and this pose was subjected to further MD coupled to binding affinity calculations (see below). The models of Ang IV in the two considered conformations, i.e., extended and with inverse γ-turn, were also used as starting points for binding affinity calculations. Finally, the cyclic peptide oxytocin was docked with GLIDE (same parameters as above), starting from its NMR structure (PDB code 2MGO). From the top ranked poses, we retained the binding mode where the binding interactions at the N terminus were in most agreement to those of Ang IV, and this pose was refined with 20 independent MD simulations of 10-ns length each.

**MD Simulations and LIE Calculations**

MD simulations were performed using the program Q (Marelius et al., 1998) with the OPLS-AA force field (Jorgensen et al., 1996). The parameters needed for the Zn$^{2+}$ ion and the ligands were retrieved from automatic parameterization performed with MacroModel, version 10.6 (Schrödinger, LLC), except for the partial charges of HA08 and HA09, which were parameterized based on similar compounds. Spherical boundary conditions were used, with a simulation sphere of 25 Å (HA08, HA09, and Ang IV) or 30 Å (oxytocin) radius centered on the same point as defined for the docking calculations. This sphere was solvated with TIP3P water molecules (Jorgensen et al., 1983) and
subject to polarization and radial constraints according to the surface-constrained all-atom solvent model (King and Warshel, 1989; Marelius et al., 1998) at the surface interface to mimic the properties of bulk water. Protein atoms outside the simulation sphere were restrained to their initial positions and only interacted with the system through bonds, angles, and torsions. The ionization states of titratable residues within 20 Å of the Zn$^{2+}$ ion, as well as the residues Lys520, Lys726, Glu767, Asp773, Arg817, Glu818, Arg820, Glu825, Arg858, Glu887, Lys890, Lys892, Glu895, Arg933, and Glu1002 were treated as ionized, whereas the remaining residues were modeled in their neutral form. With this setup the simulation sphere was overall neutral, thus avoiding the consideration of additional Born terms in the calculation of free energies. Nonbonded interactions were calculated explicitly up to a 10 Å cutoff, except for the ligand atoms for which no cutoff was used. Beyond the cutoff, long-range electrostatics were treated with the local reaction field multipole expansion method (Lee and Warshel, 1992).

During the equilibration stage, which lasted 175 ps, the system was slowly heated to the target temperature of 310 K while the initial positional restraints on all solute heavy atoms were gradually released. In the subsequent data collection phase, a step size of 1 fs was used and no restraints were applied, except for solvent bonds and angles, which were constrained using the SHAKE algorithm (Ryckaert et al., 1977). Nonbonded pair lists were updated every 25 steps, whereas the ligand-surrounding interaction energies were sampled every 50 steps. The binding mode selected for each inhibitor was subjected to 10 independent simulations with the same conditions but with different starting velocities for binding affinity calculations (see below). Here, the production phase lasted for 2 ns, where ligand-surrounding energies were collected. In parallel, reference calculations for each ligand in water were carried out using the same protocol as in the bound state.

Binding free energies were calculated using the linear interaction energy (LIE) method (Aqvist et al., 1994; Hansson et al., 1998):

$$
\Delta G_{\text{bind}}^{\text{LIE}} = \alpha \Delta U^{\text{conf}} + \beta \Delta U^{\text{el}} + \gamma
$$

where $\Delta U^{\text{conf}}$ and $\Delta U^{\text{el}}$ are the differences in the average nonpolar and polar ligand-surrounding interaction energies in the two states, i.e., free and bound. The coefficients $\alpha$ and $\beta$ are scaling parameters (Hansson et al., 1998; Almlöf et al., 2004, 2007) for the nonpolar and polar terms, respectively. In the standard LIE model $\alpha$ has a value of 0.18, whereas $\beta$ depends on the chemical nature of the ligand. The IRAP active site has a divalent zinc ion together with a cluster of carboxylates, causing large electrostatic interaction energies with the ligands. Because these interaction energies, particularly those involving the Zn$^{2+}$ ion, will be very sensitive to the force field parameters, we follow here the protocol used earlier for binding sites containing ions and treat $\beta$ as a free parameter (Mishra et al., 2012). This also allows $\beta$ to compensate for possible insufficient dielectric screening in the microscopic system. The final term $\gamma$ is a protein-dependent constant that does not affect the relative binding free energies (Almlöf et al., 2004) but is used to offset the resulting calculated energies with the experimental values. Thus, the values of $\beta$ and $\gamma$ in this system were parameterized to 0.19 and 7.30 kca/mol, respectively.

**Enzyme Assays**

**Human IRAP.** Solubilized, crude membranes isolated from HEK 293 cells (ATCC, Manassas, VA) transfected with pCI-IRAP, were used as a source of the enzyme to analyze the efficacy of the candidate compounds to inhibit its enzymatic activity. As a negative control, membranes from vector-only transfected cells were used. The enzymatic activity of IRAP was determined by the hydrolysis of a synthetic aminopeptidase substrate L-leucine-4-methyl-7-coumarinylamide (Sigma-Aldrich, St Louis, MO) monitored by the release of a fluorogenic product at excitation and emission wavelengths of 380 and 430 nm, respectively. Assays were performed in 96-well plates; each well containing 1 μg of solubilized membrane protein and 25 μM substrate in a final volume of 100 μl 50 mM Tris-HCl buffer (pH 7.4). Reactions proceeded at 37°C for 30 minutes in a Wallac Victor3 V Multilabel counter (Perkin-Elmer, Waltham, MA). Inhibitory constants ($K_i$) for each of the inhibitors were determined over a range of concentrations (0.01 to 100 μM) and were calculated from the relationship $IC_{50} = K_i \times (1 + [S]/K_s)$ with GraphPad Prism 3 software (GraphPad Software Inc. San Diego, CA).

**Leukotriene A4 hydrolase.** Recombinant human leukotriene A4 hydrolase (LTA4H; Cayman Chemical, Ann Arbor, MI) (specific activity 166 U/mg) was incubated at room temperature with 100 μM alanine-β-naphthylamide as a substrate in 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM KCl with or without increasing concentrations of an inhibitor. The fluorescence was measured at excitation and emission wavelengths of 320 and 405 nm, respectively.

**Aminopeptidase N.** AP-N (specific activity 40 U/mg; Sigma-Aldrich) was incubated with 250 μM of substrate alanine-β-naphthylamide (Sigma-Aldrich) in Tris buffered saline (50 mM Tris-HCl, 150 mM NaCl pH 7.5) at 25°C. IRAP inhibitors (1–10 μM) were added after 1 minute, and fluorescence was monitored at excitation and emission wavelengths of 320 and 405 nm, respectively.

**Primary Hippocampal Cultures**

Primary hippocampal cultures were prepared according to a previously described method (Kuehn and Banker, 2006) with slight modification. In brief, pregnant Sprague-Dawley rats (Charles River, Sulzfeld, Germany) were euthanized with carbon dioxide to allow the isolation of hippocampal neurons from mixed embryonic day 17 fetal rats. Hippocampi were chemically digested with 0.4 mg/ml trypsin (Sigma-Aldrich) for 10 minutes at 37°C, followed by mechanical dissociation with a 1-ml pipette in Hank's balanced salt solution (Invitrogen, Carlsbad, CA) containing 0.5 mg/ml trypsin inhibitor (Sigma-Aldrich). Cells were seeded on 24-well plates containing glass coverslips precoated with 50 μg/ml poly-L-lysine (MW 70,000-150,000; Sigma-Aldrich) at a density of 1 × 10^6 cells per well, and maintained at 37°C/5% CO2 in a humidified incubator. Cultures were grown in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), 0.5 mM GlutaMAX supplement (Invitrogen), and 10% (v/v) fetal bovine serum (Invitrogen) for 24 hours, after which the media were replaced with serum-free medium containing an additional 2% (v/v) B27. Partial media changes were performed twice a week. Hippocampal cells were grown for 13 days in vitro (DIV), a time when peak dendritic growth and spine formation occurs (Kuehn and Banker, 2006), before the addition of varying concentrations of inhibitors on days 14, 17, and 20. Treatments were ceased at 21 DIV and were found to have no noticeable effects on cell viability, as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. All animal experiments were approved by the Animal Ethics Committee at Uppsala University.

**Immunocytochemistry and Image Analysis**

At 21 DIV, cells were fixed with 4% (v/v) paraformaldehyde containing 0.12 M sucrose. After fixation, cells were washed in phosphate-buffered saline and permeabilized with 0.2% (v/v) Triton-X-100. Cells were blocked with 10% (v/v) normal donkey serum (Sigma-Aldrich) for 1 hour at room temperature and incubated with either mouse anti-β III-tubulin (1:500; Sigma-Aldrich), a marker of neuronal processes, or rabbit anti-drebrin (1:500; Enzo Life Sciences, Farmingdale, NY), a marker of dendritic spines, for 90 minutes at room temperature. Cells were then incubated with the appropriate fluorescent-conjugated secondary antibodies (1:500; Alexa 488 and Alexa 568; Invitrogen) for 1 hour at room temperature, rinsed with...
phosphate-buffered saline, and mounted with MOWIOL anti-fade mounting medium (Sigma-Aldrich) containing 4',6-diamidino-2-phenylindole, dihydrochloride (Merek Millipore, Bedford, MA). Images were captured using a Zeiss LSM700 inverted confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with a x63 oil immersion lens. The number of total spines, stubby/mushroom-like spines, and filopodium/thin-like spines were counted on three individual basal dendrites (primary and secondary) from 10 neurons per culture (3–6 independent cultures) using Image J 1.49e software (National Institutes of Health, Bethesda, MD). Selected segments were at least 50 μm away from the cell body, and the numbers of spines were counted on dendritic segments spanning 50 μm. Spines were defined and quantified as previously described (Garcia et al., 2010). Two populations of spines were identified based on the morphology of the spine and intensity of drebrin staining. Spines with or without a small neck that had a small and rounded spine head ≥0.7 μm in diameter, exhibiting high concentrations of drebrin, were considered stubby/mushroom-like spines, whereas spines that were typically longer, thin, and lacked a distinguishable spine head were considered filopodium/thin-like spines. All images were captured and analyzed by a researcher blinded to the treatments. For spine functionality experiments, cells were incubated with rabbit anti-vesicular glutamate transporter 1 (vGLUT1, 1:500; Abcam, Cambridge, MA), a marker of presynaptic terminals. Immunostaining controls where the primary antibody was systematically removed were performed to ensure specificity of staining.

Statistical Analyses
All data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test using GraphPad Prism, version 5 (GraphPad Prism Software Inc.). A P value <0.05 was considered statistically significant. Numerical data are represented as the mean ± S.E.M.

Results
MD simulations and LIE calculations, Ang IV, HA08, and HA09
After docking of the inhibitors HA08, HA09, and Ang IV into the IRAP active site, binding free energies (ΔGbind) were calculated using MD simulations and the LIE method (Aqvist et al., 1994; Hansson et al., 1998). For Ang IV, we considered two different docking solutions corresponding to the extended and inverse γ-turn (Ang IV-gt) conformations. In optimizing the LIE β parameter we took the average of the Ang IV interaction energies (in both the bound and free states) for the two conformations, which yields an LIE model with very close agreement between calculated and observed binding free energies (Table 1). If the two Ang IV cases are instead optimized separately, the predicted binding free energies are ~9.7 kcal/mol for the extended conformation and ~10.0 kcal/mol for Ang IV-gt. The changes in the predicted binding free energies for HA08 and HA09 are less than 0.1 kcal/mol with those models. Hence, the final calculations consistently predict HA08 to be the most potent inhibitor and also indicate that the Ang IV-gt conformation may be slightly more favorable than the extended one, although the associated errors are larger for Ang IV because of the fact that it is a larger ligand.

In comparing the extended and Ang IV-gt conformations it is clear that both of these exhibit a conserved binding mode where the peptide carbonyl group of the N-terminal residue is coordinated to the Zn2+ ion, whereas the terminal amine is fixed by three glutamate carboxylates (Glu431, Glu487, and Glu295). However, out of six residues it is only the first two that are identical between the two conformations (Fig. 3A).


<table>
<thead>
<tr>
<th>Ligand</th>
<th>Free</th>
<th>Bound</th>
<th>Enzyme Ki ± S.D.</th>
<th>ΔGobs bind kcal/mol</th>
<th>ΔGcalc bind kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA08</td>
<td>-321.9 ± 2.5</td>
<td>-32.9 ± 0.2</td>
<td>-401.9 ± 0.7</td>
<td>-43.1 ± 0.2</td>
<td>-3.3 ± 0.8</td>
</tr>
<tr>
<td>Ang IV</td>
<td>-357.8 ± 1.6</td>
<td>-37.9 ± 0.2</td>
<td>-417.9 ± 5.1</td>
<td>-66.6 ± 0.7</td>
<td>62.4 ± 17.5</td>
</tr>
<tr>
<td>Ang IV (γ-turn)</td>
<td>-346.0 ± 2.3</td>
<td>-35.9 ± 0.3</td>
<td>-409.6 ± 4.4</td>
<td>-64.6 ± 0.8</td>
<td>62.4 ± 17.5</td>
</tr>
<tr>
<td>HA09</td>
<td>-329.3 ± 1.4</td>
<td>-25.1 ± 0.1</td>
<td>-396.8 ± 1.3</td>
<td>-42.9 ± 0.4</td>
<td>242.0 ± 72.0</td>
</tr>
</tbody>
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TABLE 1
Experimental and calculated binding free energies (in kcal/mol) for the IRAP inhibitors
Calculated binding free energies are obtained with an optimized LIE model, with β=0.19 and γ=7.3 kcal/mol. Errors in calculated binding energies correspond to S.E.M. for replicate simulations.

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further stabilized by Phe550 of the enzyme (Fig. 3C). Moreover, the HA08 carboxylate group has a hydrogen bond to Tyr961 and interacts via a water molecule with the positively charged Arg929, as is the case also for Ang IV in both of its possible conformations (Fig. 3D). The C-terminal part of HA09 is generally more flexible because of its different chirality, resulting in weaker interactions with the protein.

The macrocyclic core of both HA08 and HA09 very closely mimics the conformation of Ang IV in this region and this part of the ligand is stabilized by hydrophobic interactions with Phe544 and Tyr549 (Fig. 3D). With regard to Ang IV, it was observed that its inverse \( \gamma \)-turn conformation relaxes somewhat during MD equilibration toward the extended conformation. This relaxation allows the C-terminal carboxylate group to move into a position similar to that of the extended conformation. As noted above, the interaction with Arg929 is clearly important for the affinity of Ang IV, but the carboxylate group is also largely solvent exposed in the substrate cleft of IRAP. It is further noteworthy that the C-terminal aromatic ring group of HA08 can either be seen as mimicking the proline residue in the extended Ang IV or the histidine residue in Ang IV-gt, because it overlays with both of these alternatives.

**Fig. 3.** Superimposed average structures from MD simulations. (A) The extended Ang IV (cyan) and Ang IV-gt (yellow) conformations. (B) The synthetic inhibitors HA08 (magenta) and HA09 (green). (C) Close-up of the hydrophobic packing of HA08 and HA09. (D) HA08 (magenta), and Ang IV-gt (yellow). The key residues contributing to binding are shown. The zinc ion is represented as a gray sphere.

IRAP Selectivity for Cyclic Peptides

A particularity of IRAP compared with other metallopeptidases is its unique ability to cleave cyclic peptides. We explored the binding mode of the cyclic peptide oxytocin by combining ligand docking and MD simulations. Note that, because oxytocin is a substrate of IRAP, there is no experimental data of its binding affinity, which precluded us to estimate this value computationally. Similar to the peptidic (Ang IV) and nonpeptidic (HA08, HA09) inhibitors, the cyclic part of oxytocin binds deep in the catalytic domain, contributing to the coordination of the Zn\(^{2+}\) ion (carboxyl of Cys\(^1\)) while maintaining strong electrostatic interactions between its free amine and Glu431, Glu487, and Glu295 (Fig. 4A). As it can be appreciated in the figure, the N-terminal fragment (Cys\(^1\)-Tyr\(^2\)) and the disulfide bridge of oxytocin nicely overlay with the corresponding fragments of HA08, supporting the design of this disulfide cyclized inhibitors (Andersson et al., 2010). The bioactive conformation of the cyclic fragment of oxytocin is favored by the particular conformation of the GAMEN loop in IRAP. This observation, which was already hypothesized on the basis of the two recent crystal structures of IRAP (Hermans et al., 2015; Mpakali et al., 2015) is here further supported by the results of our MD simulations, with backbone root mean square deviation values as low as 1.56 ± 0.2 Å for the cyclic fragment (Cys\(^1\)-Tyr\(^2\)) and the disulfide bridge of oxytocin nicely overlay with the corresponding fragments of HA08, supporting the design of this disulfide cyclized inhibitors (Andersson et al., 2010). The bioactive conformation of the cyclic fragment of oxytocin is favored by the particular conformation of the GAMEN loop in IRAP. This observation, which was already hypothesized on the basis of the two recent crystal structures of IRAP (Hermans et al., 2015; Mpakali et al., 2015) is here further supported by the results of our MD simulations, with backbone root mean square deviation values as low as 1.56 ± 0.2 Å for the cyclic fragment (Cys\(^1\)-Cys\(^6\)) of oxytocin and of 0.93 ± 0.1 Å for the GAMEN loop. The optimal complementarity between oxytocin and the GAMEN loop of IRAP is in stark contrast with the conformation of the same GAMEN loop found in the closest related metallopeptidases ERAP1 and ERAP2 (see Fig. 4B). Thus, our results reinforce the hypothesis that the specificity of IRAP for the cyclic peptides is, at least in part, due to the particular configuration of its GAMEN loop (Hermans et al., 2015; Mpakali et al., 2015).
Biochemical Characterization of HA08 and HA09

**IRAP.** Kinetic analysis of the inhibition of IRAP revealed that both HA08 and HA09 are competitive inhibitors (data not shown). The macrocycle HA08 exhibited an IC$_{50}$ value of 8.6 nM (8.24 and 9.04 nM) and HA09 an IC$_{50}$ value of 0.71 μM (0.68 and 0.74 μM). Hence, HA08 is a 100-fold more powerful IRAP inhibitor than HA09. Previously, the $K_i$ values for HA08 and HA09, applying a related enzymatic assay with recombinant human IRAP, were determined to be 3.3 nM and 0.242 μM, respectively (Andersson et al., 2010).

**AP-N and LTA4H.** Regarding selectivity, we found that both of the macrocyclic compounds exhibited much lower affinities for the structurally related enzymes AP-N and LTA4H. Thus, at a concentration of 10 μM for HA08, an inhibition of 36% (35 and 38%) of AP-N was encountered, whereas only 1% (0 and 2%) inhibition was observed at the same concentration with HA09. Both HA08 and HA09 were very poor inhibitors of LTA4H, showing <2% inhibition at 10 μM. The percent inhibition was benchmarked against 100 μM bestatin.

**Effect of HA08 and HA09 on Spine Morphology and Density**

The specific inhibition of IRAP activity was previously shown to enhance memory in naive rats (Albiston et al., 2008). Enhanced cognitive performance has been linked to the augmentation of synaptic connectivity, which includes changes in the number and morphology of dendritic spines.
To investigate the effect of HA08 and HA09 on spine development and their potential cognitive-enhancing effect, we used immunofluorescence to analyze spine formation in hippocampal cultures. At 21 DIV, untreated hippocampal neurons developed numerous spines (Fig. 5A), which included stubby/mushroom-like spines, a morphology associated with mature spines (Hayashi and Shirao, 1999), and filopodium/thin-like spines, a morphology associated with immature spines (Fig. 5B) (Ziv and Smith, 1996).

Immunocytochemical staining after exposure of cells to varying concentrations of HA08 at 14, 17, and 20 DIV revealed an increase in the number of dendritic spines, with a marked increase in spine density observed at 0.1 and 1 μM (Fig. 6A). After quantification, a significant increase in total spine density was observed at 0.01 (mean spine number = 15.2), 0.1 (mean spine number = 15.5), and 1 μM (mean spine number = 17.8), with HA08 inducing a 12.7 (P < 0.05), 14.4 (P < 0.05), and 30.1% (P < 0.001) increase in total spine number, respectively, compared with the vehicle control (mean spine number = 13.5; Fig. 6B). In addition, an increase in the number of stubby/mushroom-like spines was observed 0.1 and 1 μM compared with the vehicle control (0.1 μM = 21.9%, P < 0.05; and 1 μM = 42.1%, P < 0.001; Fig. 6C). These effects were not observed at

![Fig. 6. Effect of HA08 on dendritic spine number and morphology. Cultures were exposed to varying concentrations of HA08 at 14, 17, and 20 DIV. Spine number and morphology were assessed along 50 μm of dendrite in 10 neurons (3 dendrites per neuron; n = 5 independent cultures). (A) Representative images of dendritic segments depicting changes in spine density. (B) Dose-response of the percentage change in total spine number after HA08 exposure compared with the vehicle control (0.1% DMSO). (C) Dose-response of the percentage change in stubby/mushroom-like spine number following HA08 exposure compared with the vehicle control. (D) No change in filopodium/thin-like spine number was observed after HA08 exposure compared with the vehicle control. Brain-derived neurotrophic factor (100 ng/ml) was included as a positive control. Data were analyzed by one-way ANOVA followed by Dunnett’s test. All data are expressed as the mean ± S.E.M. *P < 0.05, ***P < 0.001 compared with vehicle. Scale bar = 10 μm.](Downloaded from molpharm.aspetjournals.org at ASPET Journals on October 29, 2017)
higher or lower concentrations. No change in the number of filopodium/thin-like spines was evident (Fig. 6D). The effect of HA08 was similar to the effect seen with 24-hour exposure to brain-derived neurotrophic factor (100 ng/ml), a known inducer of spine development in hippocampal neurons (Chapleau et al., 2008; Gu et al., 2008). Unlike HA08, the less potent IRAP inhibitor HA09 had no significant effect on spine density or morphology (Fig. 7, A, B, and C).

**Effect of HA08 on Dendritic Spine Functionality**

An increase in spine density can result in an increase in excitatory neurotransmission, both of which are important for memory formation (Leuner et al., 2003). Given the significant increase in mushroom/stubby spines observed after exposure to 0.1 and 1 μM HA08, we next labeled cells for the presynaptic markers synapsin1 and vGLUT1. The number of stubby/mushroom-like spines staining positive for synapsin1 or vGLUT1 were counted to determine their functional capacity (Fig. 8, A and B). When compared with the vehicle control, there was no significant change in the percentage of drebrin-positive/synapsin1-positive or drebrin-positive/vGLUT1-positive spines (Fig. 8, C and D), indicating that HA08 did not alter the ratio of functional synapses. However, because HA08 induced an overall increase in the number of stubby/mushroom-like spines, there was an overall increase in the number of synapsin1- and vGLUT1-positive spines compared with vehicle treated cells (P < 0.01; Fig. 8, E and F). Overall, these results suggest that HA08 may enhance excitatory input.

**Discussion**

IRAP is reported to be the only M1 aminopeptidase known to cleave macrocyclic rings such as those in oxytocin and vasopressin (Hermans et al., 2015). The macrocyclic HA08 that comprises a β-amino acid tyrosine residue is derived from stepwise iterative modifications of Ang IV by introduction of steric constrains by primarily 1→3 cyclizations. Proteolytic processing of peptides can lead to the formation of fragments that have very different biologic activities from those of the parent peptides (Hallberg and Nyberg, 2003; Hallberg, 2015). Hence, the hypertensive octapeptide Ang II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) is degraded to the cognitive enhancer Ang IV, which acts as an inhibitor of IRAP. The IRAP substrate oxytocin comprises a Cys-Tyr-Ile structural element in the N terminus where Cys is enrolled in a S-S bond to a second Cys, affording the large macrocyclic system of oxytocin. The Cys-Tyr bond is the primary cleavage site for IRAP. We attempted to mimic this N-terminal part of oxytocin by applying various oxidative cyclizations with cysteine and homocysteine residues in the 1→3 positions of Ang IV (Ang IV, Kᵢ = 62 nM). However, these efforts produced only poor, e.g., the 1→3 cysteine analog with a Kᵢ of > 1000 nM and the 1→3 homocysteine analog, with a slightly better inhibitory capacity, Kᵢ = 303 nM (Axen et al., 2006, 2007; Andersson et al., 2008). Oxidative cysteine cyclizations of positions 4 and 6 of Ang IV (i.e., His and Phe displaced), on the contrary, delivered a potent inhibitor with a Kᵢ = 26 nM, comprising a 11-membered ring that adopts a γ-turn and by subsequent displacement of this ring system with a simplified γ-turn mimic, a phenylacetic acid derivative was afforded. Further structural optimization led to a series of potent derivatives, among them the very potent IRAP inhibitor HA08 encompassing the 13-membered ring system (Andersson et al., 2010, 2011).

The computational docking and MD simulations presented here predict that the macrocyclic core of HA08 and HA09, as well as the corresponding part of Ang IV, have a conserved binding mode dictated by the Zn²⁺ ion and three glutamic acids. Moreover, the cyclization strategy discussed above is supported by the bioactive conformation modeled for the cyclic peptide oxytocin. The models also predict that the chirality of the synthetic inhibitors HA08 and HA09 affect and alter both the intramolecular and the ligand-enzyme hydrophobic interactions. Here, HA08 benefits from hydrophobic packing and is stabilized by Phe550, resulting in a conformation that closely mimics the natural inhibitor Ang IV.

A reduction in spine density is an early feature of many neurodegenerative diseases (Fiala et al., 2002; Bourne and Harris, 2008; van Sproosn and Hoogenraad, 2010; Penzes et al., 2011). In particular, the specific loss of stubby/mushroom spines, which morphologically have a larger spine head and functionally have stronger synapses (Nusser et al., 1998; Matsuzaki et al., 2001; Murthy et al., 2001), is believed to have a greater impact on cognitive decline (Kasai et al., 2003). Therefore, drugs that enhance spine density have been proposed for the treatment of memory disorders (Lynch et al., 2008). IRAP inhibitors have been shown to strengthen long-term potentiation (LTP) and enhance baseline neurotransmission in vitro and in vivo (Kramar et al., 2001; Wayner et al., 2008).

**Fig. 7.** Effect of HA09 on dendritic spine number and morphology. Cultures were exposed to varying concentrations of HA09 at 14, 17, and 20 DIV. Spine number and morphology were assessed along 50 μm of dendrite in 10 neurons (3 dendrites per neuron; n = 5 independent cultures). No change in total spine number (A), stubby/mushroom-like spine number (B), or filopodium/thin-like spine (C) number was observed after HA09 exposure compared with the vehicle control. Brain-derived neurotrophic factor (100 ng/ml) was included as a positive control. Data were analyzed by one-way ANOVA followed by Dunnett’s test. All data are expressed as the mean ± S.E.M. ***P < 0.001 compared with vehicle.
2001), and changes in dendritic spine architecture, such as increased spine number or morphology, have been linked to LTP (Kasai et al., 2010) and memory (Xu et al., 2009). Given this link between synaptic density, LTP, and memory, we sought to investigate the possible relationship between IRAP inhibition and spinogenesis.

Quantification of spine density and morphology revealed that submicromolar concentrations of HA08 could significantly increase dendritic spine density and alter spine morphology in hippocampal cell cultures. Treatment with 0.1 and 1 μM HA08 for 3 days over a period of a week, resulted in spines appearing more stubby/mushroom-like, a morphology typically associated with mature spines, which are believed to have strengthened synaptic connectivity (Saneyoshi et al., 2010). In contrast, HA09 did not alter total spine density or morphology compared with the vehicle control. Given that previous studies have shown that the procognitive activity of a molecule in vivo correlates well with its in vitro ability to alter dendritic spine structure (Benoist et al., 2011; McCoy et al., 2013), these results suggest that HA08 may have cognitive enhancing effects.

To further investigate the effect of HA08 on dendritic spine structure, we performed functionality studies to identify their neurotransmitter signature. Immunocytochemical staining against synapsin1, a universal marker of synaptic terminals, revealed that the majority (>90%) of dendritic spines formed active synapses after 0.1 and 1 μM HA08 treatment. Dendritic spines were also vGLUT1 positive, indicating that spines were receptive to glutamatergic signaling. Interestingly, there were fewer vGLUT1-positive spines compared with synapsin1-positive spines, suggesting that some synapses may be involved in nonglutamatergic neurotransmitter pathways, which are also known to play an important role in learning and memory processing (Jerusalinsky et al., 1997; Gong et al., 2009).
The positive effect of IRAP inhibitors on spine density and morphology may be attributed to an increase in the levels of endogenous IRAP substrates, such as oxytocin. Notably, the IRAP inhibitor Ang IV has been shown to increase levels of oxytocin in the brain in vivo (Beyer et al., 2010). The hippocampus is enriched with oxytocin receptors (Lee et al., 2015) and oxytocin has been shown to stimulate adult neurogenesis in the hippocampus (Leuner et al., 2012) and enhance LTP in the CA1 region (Lin et al., 2012). Therefore, the potential pro-cognitive effects of IRAP inhibitors may be mediated by increases in the levels and prolongation of activity of substrates including oxytocin. Oxytocin has recently been reported to enhance learning and memory, particularly in the novel recognition paradigm in rodents (Gard et al., 2012; Havranek et al., 2015). Although the effect of oxytocin on hippocampal dendritic spine density is not known, its effect on glucose uptake is documented. In cardiomyocytes, oxytocin has been shown to enhance glucose uptake via translocation of GLUT4 to the cell surface (Florian et al., 2010) and it is plausible that this effect may also occur in neurons. Glucose is known to facilitate memory in both humans and rodents (Manning et al., 1993; Korol and Gold, 1998; McNay et al., 2000). Interestingly, inhibitors of IRAP have also been shown to facilitate glucose uptake via the insulin-regulated glucose transporter GLUT4, which is found to colocalize with IRAP in neurons in intracellular vesicles (Fernando et al., 2008).

Overall, this study indicates that IRAP inhibition enhances spine density, a cellular process that has been closely associated with memory enhancement. However, further studies are needed to demonstrate direct cognitive enhancing effects of the IRAP inhibitor HA08 in vivo are needed.

Conclusion

There has been some dispute as to the target that mediates the memory-enhancing effects of Ang IV because this hexapeptide has the ability to bind to other proteins. The data presented herein provide support for the hypothesis that specific inhibitors of IRAP may induce the pro-cognitive effect by altering dendritic spine density and morphology. Competitive inhibition of IRAP activity by the 13-membered macrocycle HA08 with an IC_{50} value of 8.6 nM and that mimics the N-terminal of oxytocin and vasopressin, alters the morphology of dendritic spines in primary hippocampal cultures. The number of stubby/mushroom-like spines increased, an outcome associated with improved memory. In contrast to HA08, the structurally very similar epimer HA09 with an IC_{50} value of 710 nM and which differs only with regard to its stereochemistry at one stereogenic center, did not affect spine morphology. By applying MD simulations and the LIE method, tentative modes of binding of Ang IV, HA08 and HA09 to the human IRAP can be proposed. Notably, the calculations suggest that both the extended and the γ-turn conformation of Ang IV are favorable for binding. In combination with biologic assays, the capacities and differences in activities of the IRAP inhibitors Ang IV, HA08 and HA09 can be predicted by the calculations and such simulation methods, which will be valuable tools in design processes aimed at discovering novel IRAP inhibitors as potential cognitive enhancers.

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References


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